Process for comparative screening of substances having a pharmacological activity

The invention relates to a process for screening substances having a pharmacological activity.

Screening processes with a high throughput rate ("High Throughput Screening") and related screening technologies have become the most important processes for discovering new active substances in the pharmaceutical industry. Generally, processes of this kind are defined by the fact that a large number of substances originating from pure chemical banks, natural products or from combinatorial chemistry are tested in specific biological assays, designed in terms of the desired effect of the drug, in a most cost-effective manner and in the shortest possible time. Conventional types of assay, e.g. receptor binding or enzyme substrate assays, have already been modified relatively easily to operate by the high throughput format, which has been made necessary by the progress made with the apparatus, e.g. the methods of handling on a liquid basis, and the detection methods, combined with robot systems.

Assay systems based on living cells have hitherto been used less frequently in high throughput screens, particularly because of the difficulties encountered on standardisation, the problem of interpreting the results from the primary screening and the unsatisfactory nature of the equipment in overcoming the demands of cellular systems and long incubation times. Another reason why the use of cellular assays for high throughput screening has hitherto been regarded as being less attractive is in the

large outlay involved in primary screening, i.e. the first screening process, to distinguish between toxic, non-specific or non-selective effects of the substance or to clarify the mechanisms of activity occurring in the cell when the cellular system responds to a substance, i.e. in the event of a "hit".

With the aid of the highly developed techniques of molecular biology, biological target molecules the activity of which is supposed to modulate the drug to be identified can be incorporated in eukaryotic cells, particularly mammalian cell lines. In addition, there are various methods available for detecting the desired effect in the target cell, e.g. the production of a new protein as the result of the stimulation of a specific receptor, the activation or increase in measurable events coupled to the target mechanism, e.g. the expression of a reporter gene product such as luciferase or Green Fluorescent Protein (GFP) or influencing key events which occur in the cell after receptor activation, e.g. apoptosis.

The characteristics of a good cell-based assay are as follows:

a) The "signal/noise" ratio, i.e. the ratio of maximum change and normal level of the measuring signal, has to be a favourable one, i.e. the background signal should be low enough to detect induction of the event to be measured with high sensitivity but at the same time high enough to be able to determine the detection limits with respect to the negative controls. b) The signals measured must be reproducible and stable over a certain period of time. c). The test cells must be relatively easy to culture and resistant to treatment in the assay. d) The biological target molecule introduced into the cells and the regulation mechanisms coupled thereto must be stable over

a fairly long period (several months). e) The properties of the cell must remain the same over a fairly long period of time; i.e. the cell lines used must also be stable *per se*.

Satisfying these requirements constitutes the basis for a screening system the chief advantage of which is that living cells prepare a substrate which provides the substances to be tested with the target molecule which they are supposed to, in the last analysis, modulate in the living organism, being potential therapeutic drugs, in the correct biological environment. One example of a test substrate consists, for example, of cells of a cell line which contain a cloned receptor and the signal transmission pathway associated therewith, coupled to a suitable reporter system.

High throughput strategies are highly efficient if the information obtained in the primary screening can be maximised. At the same time, this means that the outlay connected with the primary screening which consists in qualitatively differentiating the "hits" obtained must be kept to an absolute minimum. With the screening throughput rates now achievable, of the order of a million substances per annum, a "hit" rate of more than 1% results in unacceptable expenditure. Typical hit quotas of more than 1% and the complex secondary assays connected with them mean that, in spite of the theoretical advantages of cellular assays, the user will frequently fall back on alternative screening formats such as receptor binding or enzyme substrate assays.

The objective underlying the present invention is to maximise the theoretical advantages of cellular assays and provide a process which does away with the disadvantages

that occur in cellular assays of the high throughput format.

The concept of "parallel screening" is generally applied to the logistic mechanism in screening whereby a number of different assays or assay formats are carried out with the same arrangement of equipment under the control of a robot.

The present invention was based on the idea of further developing this approach for comparative screening, by simultaneously charging a number of different cellular assay formats from the same supply of substance.

The present invention relates to a process for determining the pharmacological activity of a substance on the activity of different biological target molecules by applying the substance to test cells which contain one or more biological target molecules and determining the effect of the substance on the activity of the target molecules. The process is characterised in that a defined quantity of a test substance, in one operation, is

a) applied to test cells with the same basic biological constitution which differ in that they contain one or more different biological target molecules; and/or

b) is applied to test cells which contain one or more biological target molecules, the cells differing in that they have different basic biological constitutions, and in that

i) the effect of the substance on the biological target molecule or molecules is measured by means of a detection system coupled to the activation of the target molecule; and/or

ii) the effect of the substance on different regulatory mechanisms triggered by the activation of the target molecule is determined by measuring the effect using a plurality of detection systems each coupled to the different regulatory mechanisms,

and in that the effects of the test substance on the different test cells or the effects determined by different detection methods are compared directly with one another.

The test cells used in the process according to the invention and containing one or more specific biological target molecules constitute a defined cellular substrate onto which the test substance in question is applied. The substrate (in case a) cells with identical biological background and different target molecules; in case b) cells with different biological background (basic constitution) and identical target molecule) generally consists of a number (up to about 10^5) of identical cells each located in a well of a culture plate. If desired, single cells may also constitute the substrate and be acted upon by the substance if the detection system is sensitive enough, e.g. if, in the event of measuring optical signals, the conditions in the apparatus are able to amplify the signals accordingly by physical methods.

The main feature of the invention consists in the fact that the same substance is applied to a number of different cellular substrates in one operation. Each substrate represents an individual assay type. If different detection systems are used to investigate the effect of the test substance on different regulatory mechanisms of the same target molecule, there are different assay formats based on the same substrate.

The process according to the invention has the advantage of minimising the variables which are crucial to evaluation by the use of different assays or assay formats whilst at the same time using identical logistics (identical source of substance). The process also has the advantage that the results obtained in a single operation in the various assays can be compared and evaluated according to strict criteria.

The step in which a defined amount of a test substance is applied to the cells is generally extended to a number of substances in high throughput screening, i.e. a number of substances, possibly each in several dilutions, are applied in parallel to one or more sets of cellular substrates, each set constituting a group of different assays or assay formats based on the same starting cell.

After other possible screening steps following the primary screening, in which the test substance or substances is or are applied to another set of cellular substrates, the "hits" obtained can be subdivided into a number of categories, e.g. according to the specificity of their effect on only one cell type, non-specificity in all the cell types used, or specificity in some but not all of the cell types tested, according to their specificity on only one signal transmission pathway coupled to the target molecule.

The cells are generally mammalian cells, particularly human cells; however, other eukaryotic cells may also be used as test cells, especially yeast cells.

The starting cells for the test cells are conveniently cells which are easy to cultivate and which have a stable genotype. It is possible to use cells which express the

target molecule in question endogenously. However, it is preferable to use cells which do not express the target molecules in question at all or express them only slightly. The cells are transformed by conventional transfection methods with the DNA coding for the biological target molecule, e.g. with receptor DNA (cf. for example Potter et al. 1984; Felgner et al., 1987); the electroporation, calcium phosphate precipitation or lipofection methods are preferred.

The cells are used on the one hand to produce test cells by forming the starting substrate for receiving the DNA coding for the target molecule, and on the other hand they are used as control cells to check whether a signal measured by a detecting system can actually be attributed to the effect of the test substance. If the substance generates a signal in the test cell but no signal in the starting cell used as the control cell, the effect detected by means of the signal can be attributed to the test substance. If the control cell also emits a signal, the substance (also) influences a cellular process which is independent of the activation of the target molecule; the control measurement corresponding to this signal must be taken into account when evaluating the measurement.

With a view to carrying out the method of measurement used as simply as possible, cells are used which satisfy the prerequisites for this purpose. For test cells in which proliferation or apoptosis is measured, it is possible to use both adherent cells and cells which grow in suspension and the survival of which in culture is strictly dependent on the presence of certain growth factors. If these growth factors are removed, the cells stop growing or initiate apoptosis within a few hours. For test cells based on the measurement of gene expression, it is preferable to use cells which adhere well, e.g.

fibroblasts, epithelial cells, endothelial cells, etc., as this method of measurement requires a washing step.

If according to embodiment a) of the process according to the invention cells are used having the same basic biological constitution, these are cells of the same type, i.e. cells isolated from a certain tissue and defined *inter alia* by molecular-biological, morphological, biochemical and immunological parameters. Preferably, the test cells used are clonal cells, generally cells of a permanent cell line. These cells originate from a single original cell and have an identical genotype.

For the embodiment of the invention according to b) cells having a different basic biological constitution are, by definition, not only cells of different types but also cells of the same type but with a different state of differentiation or activation. In this way, for example, the effect of a test substance on normal cells can be compared with its effect on tumour cells coming from the same tissue, or the effect of the substance on cells of an established cell line can be compared with the effect on primary cells of the same origin, thus making it possible to assess the predictive ability of screening based on permanent cell lines.

Biological target molecules are those molecules whose biological activity is supposed to be influenced by the test substance in order to block an unwanted reaction of the cell which is responsible for a pathological effect, this reaction resulting from this activity.

Theoretically, target molecules may be any of the proteins or fragments or mutants thereof which naturally occur in mammalian cells, particularly human cells, which are constitutively active or the activation of which by an

extracellular signal (the natural ligand) brings about a reaction of the cell which can be measured directly or indirectly in any form. The influence on this reaction which occurs upon inhibition by a test substance is thus also measurable, making it possible to obtain automatic evaluation when the process is used as a robot-controlled high throughput process.

Biological target molecules are components of various cellular, *inter alia* metabolic or receptor-coupled signal transmission pathways; in theory, these components may originate from any section of the signal transmission cascade.

Examples of biological target molecules are representatives of the major group of receptors, particularly receptors for growth and differentiating factors including their subtypes. Examples of receptors include receptor tyrosine kinases (EGF receptor, PDGF receptor, etc.); serine/threonine kinases such as the TGF-β receptor, integrin receptors; receptors of class LIF, oncostatin M, CNTF, etc. (gp130); receptor phosphatases; receptors for cytokines (interleukins, interferons, etc.) and G-protein-coupled receptors which are able to couple to the phospholipase C-signal transmission pathway or the adenylate cyclase signal transmission pathway, e.g. neuropeptide Y receptors (also PYY, NPP) or neurokinin receptors ($NK_{1,2,3}$).

A process for screening substances with a modulating effect on receptor-dependent cellular signal transmission pathways and its application to various receptors is described for example in WO 93/11257, to the contents of which reference is hereby made. The principle of the process is based on the use of test cells which contain a reporter gene under the control of a regulatory sequence

which responds to a change in the concentration of a messenger substance of a receptor-dependent signal transmission pathway (sensor DNA) and the receptor DNA in question. If the test substance is applied to the test cells, the effect of the test substance on the target molecule, i.e. the receptor in question or an element of the signal transmission pathway coupled to this receptor, takes the form of a change in the expression of the reporter gene. According to embodiment a) in conjunction with i) of the process according to the invention, for comparative screening on the basis of this assay principle, identical starting cells are transformed with identical reporter gene construct (sensor DNA) and with different receptor DNA, thus making it possible to make a direct comparison of the effect of the test substance on the activation of signal transmission pathways of different receptors or receptor subtypes.

According to alternative b) in conjunction with i), cells of different types are transformed with identical receptor DNA and identical sensor DNA in order to investigate the effect of the test substance on the signal transmission pathway coupled to the receptor in question in various cell types. This is particularly interesting when searching for a drug which has selectivity for a certain cell type, i.e. when the substance is intended to influence the signal transmission pathway only in a certain type of cell but not in other types of cell, e.g. the signal transmission pathway of the IL-5 receptor which is expressed on eosinophils and certain T-cells but not on other cells.

The activation of a receptor or receptor subtype can, as the result of complex intracellular interactions, initiate the activation of more than one signal transmission pathway/effector system, e.g. if a G-protein-coupled

receptor is able to interact with more than G-protein variant. For example, the angiotensin AT_1-receptor couples with the phospholipase C and adenylate cyclase signal transmission pathway, whereas the AT_2-receptor couples with the effector system of cyclic GMP. Another example is the prostanoid receptor EP_3 which couples to the phospholipase C and adenylate cyclase signal transmission pathway, whereas other prostanoid receptors couple only to the adenylate cyclase signal transmission pathway or, in one case, a nuclear receptor (PPARγ) is activated directly. If the cause of pathological changes in the cell lies in disruption of these interactions, it may be useful to discover drugs which act specifically on only one of the effector systems. In this case, the embodiment of the process according to a) in conjunction with ii) can be used. If for example one signal transmission pathway is the phospholipase C-effector system which when activated causes the messengers inositol-1,4,5-triphosphate (IP_3) and diacyl glycerol (DAG) to be formed, and the other is the adenylate cyclase effector system in which the messenger substance cAMP is formed, two test cells transformed with the same receptor can be used, which differ in the sensor DNA, one of them responding to the IP3/DAG concentration and the other responding to the cAMP concentration. Examples of suitable sensor DNA constructs can be found in WO 93/11257. The invention makes it possible in this case to state directly in one screening operation whether a substance has specificity for only one signal transmission pathway.

Alternatives ai) and aii) may also be combined in one screening process.

Other examples of target molecules are intracellular components of signal transmission pathways, e.g. protein

kinases (Src, Raf, MAPK, JAK), adaptor molecules (GRB, Sos) or other elements of signal transmission pathways such as Ras.

Target molecules may also be components located further downstream in the signal transmission pathway, such as elements of inducible gene expression (ligand-regulated transcription control), particularly transcription factors, e.g. Myc, Fos, Myb, NF-κB, AP-1, STAT, Ets-proteins and/or the co-factors thereof, or molecules which participate in cell apoptosis (Bcl-2, p53 mutants or the proteases which regulate apoptosis), as well as kinases, phosphatases or GTPases. The target molecules may also be intracellular hormone receptors such as steroid receptors or retinoid receptors. The skilled person will be familiar with such elements; an overview of inducible gene expression and the components involved therein can be found in the relevant textbooks, e.g. "Inducible Gene Expression", 1995.

The target molecules may be identical to the proteins naturally occurring in the cell; however versions of these proteins which have been mutated in various sections or which have been changed, e.g. shortened, compared with the Wild type in certain sections may also be used. Thus, for example, it is possible to investigate the influence of a test substance on certain domains of the target molecule, e.g. on the transactivation domain or on a section of the protein which is responsible for interaction with a co-factor.

The effect of a test substance on the biological target molecule is perceived by the cell as a signal that leads to different reactions of the cell depending on the cell type. Such reactions, e.g. a change in the intracellular calcium level, phosphorylation or dephosphorylation of

various proteins, transcription or expression of genes, DNA synthesis, cell division or cell differentiation (morphological changes, expression of marker genes), apoptosis, chemotaxis, cell adhesion, are undesirable when they are responsible for pathological conditions.

Using the process according to the invention it is possible to identify test substances cell-type-specifically even during the first screening by their effect on unwanted reactions of these kinds.

Theoretically, all the cellular reactions triggered by activation of the biological target molecule can be determined directly or indirectly by physical-chemical methods; the measurement obtained in the detection system correlates with the effect of the test substances on the target molecule.

Frequently, the cellular response to growth factors results in cell division (proliferation) or in the prevention of programmed cell death (apoptosis). Both the influencing of proliferation and also apoptosis as a result of the effect of the test substance can very easily be measured within the scope of fully automated screening.

In a preferred embodiment of the invention the process is based on the measurement of proliferation.

A test system based on the measurement of proliferation may be designed as follows, for example: cells which proliferate depending on growth factor are put in, then dilutions of the test substances are added and the test cells are incubated for a certain length of time. Then the cells are mixed with an assay reagent and after further brief incubation they are measured in a microtitre plate photometer. The incubation period is designed so

that test cells from which the growth factor has been withdrawn and test cells incubated in the presence of growth factor have a significant difference in the number of cells in this length of time.

Another method of detection preferably used within the scope of the present invention is based on measuring the expression of reporter genes.

A reporter gene is defined by the fact that its expression product can be detected and quantified by the measurement of a signal the magnitude of which is proportional to its concentration. Within the scope of the present invention, any reporter genes whose expression can be measured with a high degree of sensitivity in an automated assay may be used.

Examples of reporter genes are the alkaline phosphatase gene and the β-galactosidase and β-glucuronidase gene; the reactions catalysed by these enzymes are monitored using established fluorescence assays (Wieland et al., 1985; Kricka, 1988), and also chloramphenicol-acetyltransferase (CAT; Hartmann, 1991).

A reporter gene which is preferred within the scope of the present invention is the gene coding for Photinus pyralis luciferase (firefly luciferase) (De Wet et al., 1987). This enzyme has the advantages that with its substrate luciferin and with the addition of ATP it produces bioluminescence in a high yield, which can be measured by established automatable methods, and this enzyme is not produced endogenously by mammalian cells. Moreover, luciferase has a relatively short half-life *in vivo* and is also not toxic in high concentrations (Hartmann, 1991; Brasier et al., 1989). Measuring the activity of firefly luciferase by bioluminescence is one of the most sensitive

methods of determining an enzyme. For this reason and because of the absence of luciferase activity in normal mammalian cells, this enzyme is particularly suitable as a reporter gene (Subramani and DeLuca, 1987). Another preferred report gene is Green Fluorescent Protein (GFP; Inouye and Tsuji, 1994; Chalfie et al., 1994).

Reporter genes may be used *inter alia* to detect the transcription of genes triggered by the effect of the test substance:

The stimulation of a number of different signal transmission pathways ends in the transcription of a group of genes, e.g. the so-called "immediate early genes" such as c-fos, c-jun, c-myc. Thus, by measuring the expression of these genes, it is possible to demonstrate the effect of test substances on the activation or inhibition of various signal transmission pathways.

Direct measurement of gene expression, e.g. using antibodies against the expression products, is technically laborious and cannot generally be used for automatic screening. By contrast, the expression of selected genes can be determined indirectly by a relatively simple method. The effect of the test substance on the target molecule is determined by measuring the expression of a reporter gene (e.g. the luciferase gene) which is under the control of the regulatory sequence of the original gene (e.g. c-fos), instead of measuring the expression of the original gene, i.e. the gene which is naturally expressed at the end of the intracellular signal cascade triggered by the activation of the target molecule. The expression levels for the reporter gene are directly proportional to the effect of the test substance on the target molecule. A screening process of this kind based on transcription modulation which is suitable for use in

the high throughput format is described in WO 92/13063, the contents of which are hereby referred to.

The various detection systems used within the scope of the present invention either simultaneously in one step or consecutively in subsequent screening steps may differ on the one hand in the detection principle itself (e.g. reporter gene expression as against apoptosis) and/or in the design of the detection system. This makes it possible to give differential statements as to the effect of a test substance on different regulatory mechanisms which result in different, in some cases pathological, effects (e.g. the use of different sensor DNA molecules which respond to different effector systems or contain promoters of different target genes). Thus, the process according to the invention opens up the possibility of differentiation in terms of the desired prevention of a certain unwanted biological effect. If for example one wishes to find a substance which inhibits the proliferation of a certain cell type but not differentiation, and it is known that these two reactions are evoked in the same cell by activation of different regulatory mechanisms of the same target molecule, the effect of the test substance can be assigned to one of the two signal transmission pathways by a suitable assay design. For this purpose, for example, the effect of the substance on the same cell type can be determined in a comparative screening with two different detection systems each of which responds to a different regulatory mechanism. If it is known that the activation of a certain regulatory system is correlated to a certain undesirable reaction in the cell, e.g. if the activation of a certain signal transmission pathway leads to apoptosis, the effect of the test substance on apoptosis can be determined not only directly by measuring apoptosis but also indirectly by means of a reporter gene system

coupled to this signal transmission pathway. In this case, the expression of the reporter gene product as a consequence of the effect of the test substance bears witness to the fact that the substance would influence apoptosis for the selected cell type.

The process according to the invention also makes it possible to arrive rapidly and efficiently at a pronouncement regarding a target molecule which is already present in cloned form but the function of which is wholly or partially unknown, e.g. a particular receptor subtype. If a test cell transformed with the receptor of unknown function is used, together with a series of test cells of identical basic biological construction which contain other receptors of known function, in an assay the detection system of which detects the influence of test substances on a biological activity either directly or indirectly via a reporter gene system, comparison of the effect of the test substances on the test cells containing various receptors makes it possible to comment on the function of the receptor whose function has hitherto been unknown.

Within the scope of the present invention, cells of a growth factor-dependent cell line were transformed *inter alia* with different functional biological target molecules the activation of which plays a part in tumour progression, and used as substrate in a High Throughput Screen. Each group of cellular test substrates consisting of cells transformed with one of the biological target molecules was acted upon, several times over, with individual substances from a series of test substances and the proliferation of the cells was measured. The target molecules used were the EGF (Epidermal Growth Factor) receptor, the HER2 receptor, the receptor (KDR) for the vascular endothelial cell growth factor (VEGF), the

MET-HGF (Hepatocyte Growth Factor) receptor and activated Ras proteins.

These target molecules were chosen on account of their significance as potential intervention sites in tumour therapies:

The EGF receptor (EGF-R) is credited with playing an important part in the formation of various tumours of epithelial origin. This type of receptor is frequently constitutively activated in tumour tissues, specifically by overexpression, gene amplification, autocrine stimulation of the mutations. In the light of this observation, EGF-R is a potentially important point of attack in tumour therapy. Apart from EGF-R-specific monoclonal antibodies and toxin-charged ligands, some selective inhibitors of EGF-R-associated tyrosinekinase have hitherto been described. In the search for new inhibitors of EGF-R, cellular test systems have been developed within the scope of the present invention based on FDC-P1 cells which have been genetically altered so as to express functional human EGF-R. Since the FDC-P1 cells are IL-3-dependent, the proliferation of these cells can be stimulated either by murine IL-3 or by EGF (which may replace IL-3 in cells which express the EGF receptor ectopically) (von Rüden, T. et al., 1988; Pierce, J. H. et al.,1988). If these cells are cultivated entirely without growth factor, the cells die within 24 hours (apoptosis). The same is true of cells incubated in the presence of EGF and selective inhibitors of EGF-R. The proliferation or apoptosis rate depends on the concentration of the inhibitor. In order to check for non-specific toxicity of the test substances the influence of the test substances on the IL-3-dependent proliferation can be measured. These cells are suitable for screening for selective inhibitors of EGF-R.

Like the EGF receptor, the HER2 receptor is connected with the formation of epithelial tumours. As with the EGF receptor test cell, in the tests according to the present invention a test cell was set up, based on the parental cell line FDC-P1 for the HER2 receptor. Since the HER2 receptor unlike the EGF receptor normally does not transduce any mitogenic signals, being a homodimer, for setting up the test cells an HER2 mutant was used wherein the amino acid valine in position 654 was replaced by the amino acid glutamic acid. This amino acid exchange corresponds to the mutation in the *neu*-oncogene in the rat (Weiner, et al., 1989; Suda, et al., 1990) and means that the HER2 receptor is constitutively active even without ligands and without the formation of heterodimers. Ectopic expression of the mutated receptor in FDC-P1 cells induces IL-3-independent proliferation. Like the EGF-R-test cell, the HER2-test cell is suitable for finding selective inhibitors of the receptor.

The HGF receptor (c-met) thus plays a part both in tumour formation and, as a result of the ubiquitous presence of its ligand HGF in the surrounding connective tissue, in tumour invasion and metastasis. The HGF receptor is frequently overexpressed and/or amplified in tumour tissues. Up till now, no selective inhibitors of HGF-R-associated tyrosinekinase have been described. Within the scope of the present invention, in the search for inhibitors of HGF-R on the one hand analogously to EGF-R a cellular test system has been developed based on FDC-P1 cells which have been genetically altered so as to express functional human HGF-R. These cells require either IL-3 or HGF in order to survive. On the other hand KB cells which express human HGF-R (and human EGF-R) are stably transfected with a reporter gene so that activation of the receptor with HGF (or EGF) causes induction of the

reporter gene product, i.e. luciferase, which is easy to measure.

An important prerequisite for tumour growth is the blood supply. This is ensured by the formation of new blood vessels in the tumour, neoangiogenesis. For this, in an oxygen deficiency tumour cells produce the messenger substance VEGF which, after binding to the corresponding receptor "KDR", which is a receptor-tyrosinekinase (RTK), induces the proliferation and migration of the endothelial cells towards the tumour. The blockade of neoangiogenesis by inhibiting the VEGF receptor KDR is therefore an important approach in tumour therapy. Analogously to the test cells for EGF-R and HER2 test cells were prepared for the VEGF receptor KDR. Expression and activation of this RTK leads to IL-3-independent proliferation and is therefore suitable for screening for selective inhibitors.

Mutations of the Ras proteins H-Ras, K-Ras and N-Ras, which lead to constitutive activation of Ras, are found in many human tumours (Bos, 1988; Kiaris and Spandidos, 1995). The occurrence of Ras-mutations is therefore regarded as an important step in tumour formation, and consequently Ras-mutants are deemed to be target molecules in the treatment of tumours. Various Ras-inhibitors, such as monoclonal antibodies (Furth et al., 1982), dominant-negative mutants (Stacey et al., 1991; Quilliam et al., 1994), antisense RNA or inhibitors of the Ras-farnesyltransferases (Kohl et al., 1993; Kohl et al., 1994; Kohl et al., 1995), have confirmed the accuracy of the therapeutic principle of using Ras-mutants as points of intervention in the treatment of tumours. The inhibitors currently available are of only limited suitability for therapeutic purposes, however, since Ras-specific inhibitors (antibodies, dominant-negative mutants or antisense RNA) under *in vivo* conditions cannot be

introduced into all tumour cells in sufficient concentrations, and on the other hand the farnesyltransferase-inhibitors are not selective for mutated Ras, but will also influence the posttranslational modification of normal Ras, as well as other cell proteins, such as e.g. lamin A and B, skeletal muscle phosphorylasekinase, and retina proteins such as transducin and rhodopsinkinase. Moreover, K-Ras, which is most frequently mutated in human tumours, is also a substrate for the posttranslational modification by geranylgeranyl-transferase, which is only inadequately blocked by the farnesyltransferase inhibitors (James et al., 1995; Lerner et al., 1995; Manne et al., 1995). The test cells developed within the scope of the present invention can be used to search for low-molecular inhibitors of the activated Ras proteins. Within the scope of the present invention FDC-P1 cells were also used as test cells for potential Ras-inhibitors. Ectopic expression of the activated Ras-mutants K-Ras^{Val12} (McCoy et al., 1984) and H-Ras^{Val12} (Reddy et al., 1982) induces IL-3-independent proliferation of the test cells (Fig. 1 and 2). The use of a Ras-inhibitor known from the literature, e.g. the farnesyltransferase inhibitor L-739.749 (2(*S*)-{2(*S*)-[2(*R*)-amino-3-mercapto]propylamino-3(*S*)-methyl}pentyloxy-3-phenylpropionylmethionine sulfonemethyl ester; Kohl et al., 1994)) demonstrated the dependency of the test cells on the presence of the activated Ras proteins (Fig. 1 and 2). The cells used within the scope of the present invention are suitable for searching for low-molecular Ras-inhibitors and can be used in a High Throughput Screen.

In order to check the specificity of potential Ras-inhibitors control cells may be used which have been made IL-3-independent by the expression of activated proteins provided after Ras in the Ras-signal transduction pathway

and thus render cell growth independent of Ras (e.g. the signal transmission pathway which runs via Raf, MEK and ERK (= MAP Kinase) or the one which runs via Rac and Rho - optionally in combination with a second signal such as e.g. c-myc). A cell line of this kind is FDC-P1/J2, prepared by infection with a retrovirus which brings about the expression of activated v-raf and v-myc (Rapp et al., 1985). In addition to the activity of the abovementioned target molecules, overexpression of the Bcl-2 protein is regarded as one of the causes of certain malignant diseases, such as e.g. leukaemias (Reed, 1994), and furthermore overexpression of this protein is also described in tumours of epithelial origin (McDonnell, et al., 1992; Pezzella, et al., 1993; Lu, et al., 1993). There is a clear connection between Bcl-2 levels and resistance to chemotherapeutics (Kitada, et al., 1994; Miyashita and Reed, 1992). The molecular mechanism of activity of Bcl-2 is the prevention of programmed cell death (Reed, 1994). Bcl-2 thus counts as a potentially important site for attack in the treatment of certain leukaemias and solid tumours. FDC-P1 Test cells which overexpress Bcl-2 may be used for screening for low-molecular inhibitors of Bcl-2 protein, for example; these cells survive even in the absence of IL-3.

Substance "hits" obtained with the aid of a screen based on these target molecules can be classified differentially in a single step, even during the first screening, according to their activity on the target molecules which are involved in the tumour progression as the result of various mechanisms. Thus it is possible to find substances for the therapy with a very specific activity.

Apart from the cells designated FDC-P1 used in embodiment 1 of the present invention, the preparation of which was

described by Dexter, T. M. et al., 1980, in which screening was carried out for potential tumour therapy drugs, other growth factor-dependent cells may be used for screening based on the measurement of proliferation as a detection system; e.g. the cell line 32D described by Pierce, J. H. et al., 1988, or the cell lines designated BAF-B03 or DA-1 described by Shibuya, H. et al., 1992, or Alexander, W. S. et al., 1991.

The assay system may be constructed as follows, for example:
The cells are placed in 96-well microtitre plates in the presence of the corresponding growth factors. Then, e.g. using a pipetting robot such as Tecan RSP5052, dilutions of the substances to be tested are added and the test cells are incubated for a certain length of time under conventional cell culture conditions. Then the cells are mixed with assay reagent, e.g. the MTS-based "Cell titer non-radioactive proliferation assay" (Promega) and after further brief incubation, measured in a microtitre plate photometer (e.g. SLT Spectra Shell). The incubation period must be such that test cells deprived of growth factor and test cells which are in the presence of growth factor exhibit a distinct difference in the number of cells during this period. Substances which specifically inhibit a receptor tyrosinekinase (RTK) should not have any effect in cell lines which are dependent on a different RTK.

Another assay used within the scope of the present invention is based on the human epithelial cell line KB4 into which the luciferase gene has been introduced under the control of the human fos-promoter. These cells endogenously express the human EGF receptor, the human HGF receptor as well as some other receptor tyrosinekinases. Treatment of the cells after removal of serum with a ligand of these receptor tyrosinekinases leads to

activation of the fos promoter and expression of the luciferase gene, the activity of which is proportional to the strength of the activation signals over a certain range. A corresponding test system for High Throughput Screening can be set up as follows: the cells described above are placed in serum-reduced medium in 96-well microtitre plates. Then dilutions of the substances to be tested are added using a pipetting robot (e.g. Tecan RSP5052) and the luciferase gene under fos control is induced with the corresponding growth factor. The test cells are incubated for a certain length of time under conventional cell culture conditions. Then the cells are washed with a buffer solution and mixed with an assay reagent which lyses the cells and contains the components luciferin and ATP needed for the luciferase reaction. After brief incubation the luciferase signal is measured in a microtitre plate luminometer (SLT Spectra Shell; Wallac Microbeta). A specific RTK inhibitor reduces the luciferase signal in the corresponding test cell, but has no influence on the c-fos promoter activity in other test cells.

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Fig.7: Selective inhibition of the proliferation of an EGF-dependent cell line by an EGF-R inhibitor

Example 1

Proliferation assay of the High Throughput Format for screening substances with an anti-tumour activity

a) Preparation of a test cell for activated H-Ras^{Val12}

i) Expression vector for human H-Ras^{Val12}

Suitable vectors for the ectopic expression of activated human Ras proteins are retroviral vectors such as e.g. pGD (Daley et al., 1990), pLXSN (Miller and Rosman, 1989), or expression plasmids with strong eukaryotic or viral promoters such as e.g. SV40 or cytomegalovirus (CMV), under whose transcriptional control the cDNA or the gene for an activated Ras protein are placed. The test cell was

prepared using the expression vector pGDV12ras (Tanaka et al., 1994) which was brought under the control of the murine retroviral LTR by cloning the oncogenic form of human cDNA for c-H-ras^{Val12} into the EcoRI site of the retroviral vector pGD (Daley et al., 1990). The pGD vector additionally contains the neomycin-phosphotransferase gene under the control of a thymidinekinase promoter from plasmid pMC1neo (Stratagene), which, with neomycin analogues (G-418), makes it possible to select cells which have the expression construct stably integrated in their genome.

ii) Transfection

FDC-P1 cells (Dexter et al., 1980) were placed in IMDM medium (BioWhittaker), combined with 10% heat-inactivated foetal calf serum (FCS), 2 mM glutamine, 50 μM ß-mercaptoethanol and 1% IL-3 and incubated at 37°C in 5% CO_2. 10^7 cells per transfection were centrifuged off for 5 min at 1200 rpm at ambient temperature (Heraeus Minifuge), washed once with serumfree RPMI-1640 medium, and suspended in 1 ml serumfree medium. 800 μl of this cell suspension were mixed with 20 μg of plasmid pGDV12ras in an electroporation dish and electroporated with a single current surge of 280 V, 980 μF and 1000 ms (Progenetor II, Hoefer Instruments). The cells were diluted with 1% IL-3 in 10 ml of culture medium and incubated at 37°C, 5% CO_2.

Two days after transfection the cells were diluted 3 times with fresh medium and 250 μg/ml of G-418 were added to select for neomycin resistance. On the following days the G-418 concentration was increased to 500, 750 and 1000 μg/ml. Dead cells were removed by centrifugation using a Ficoll gradient. For this the cells were first concentrated by centrifugation (5 min, 1200 rpm) in a small volume and layered over Ficoll-Paque (Pharmacia) in

a centrifuge test tube. After centrifugation (15 min, 2500 rpm, 20°C) and free running from the centrifuge the live cells which had collected on the medium/Ficoll intermediate phase were pipetted off, washed once with medium and transferred into fresh medium.
Eight days after the start of G-418 selection, the IL-3 independent cells began to be concentrated by reducing the IL-3 concentration step by step. After the cells had been cultivated for one week in 0.0005% IL-3, they were separated by seeding in semiliquid Methocel medium. Cell colonies which formed and were well separated from other cells were isolated, transferred into 96-well plates with 100 µl medium (without IL-3) and subsequently expanded. One of the resulting IL-3-independent stable cell lines which exhibited good growth properties was named FHRV12cc21.

iii) Characterisation of the cells

Detection of H-ras^{Val12} expression
FHRV12cc21 and untransfected FDC-P1 cells were grown in IMDM medium with 10% FCS and 1% IL-3. In order to detect the inhibition of the posttranslational modification of H-ras^{Val12} by farnesylation the cells were incubated for 48 hours with 5 µM of the protein-farnesyltransferase-inhibitor L-739.749 (Kohl et al., 1994). The cells were washed with PBS and lysed in PBSTDS (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulphate in PBS) 5 min at 4°C. The cell lysates were clarified by centrifugation (20 min 14,000x *g*, 4°C). Ras proteins were immunoprecipitated by the addition of 1 µg monoclonal rat antibodies Y13-238 (Oncogene Science) and Protein-G Plus / Protein-A Agarose (Oncogene Science) overnight at 4°C. The precipitates were washed twice with PBSTDS buffer and then extracted in SDS-sample buffer with 3% ß-mercaptoethanol. The proteins were separated in a 20% SDS-polyacrylamide

gel, transferred onto an Immobilon membrane (Millipore) and after saturation of the membrane with 10% FCS, 1% bovine serum albumin, 1% Tween-20 in PBS, shown up by successive incubation with a Ras^{Val12} specific monoclonal antibody (pan-Ras^{Val12}, Oncogene Science) and a secondary anti-mouse-IgG - alkaline phosphatase conjugate by colour reaction with NBT and BCIP (Promega). A clear overexpression of H-Ras^{Val12} in FHRV12cc21 cells was demonstrated, compared with the untransfected FDC-P1 control cell and inhibition of the Ras farnesylation by L-739.749. The cells treated with L-739.749 contained more than 50% of the Ras protein in the unmodified (non - farnesylated) form, which therefore migrates more slowly in the SDS gel. (The detection of the overexpression of H-ras^{Val12} in FHRV12cc21 cells and the inhibition of Ras-farnesylation by L-739.749 are shown in Fig. 3. After treatment with (+) or without (-) 5 μM L-739.749 for 48 h in the presence of 1% IL-3, Ras protein was immunoprecipitated from cell lysates with anti-Ras antibodies and after electrophoretic separation in an SDS-polyacrylamide gel and membrane transfer, detected with anti-Ras^{Val12} antibodies. The name "ras^{Val12} Std." denotes a standard K-Ras^{Val12} preparation (Oncogene Science)).

After treatment with (+) or without (-) L-739.749 for 48 h in the presence of 1% IL-3 Ras protein was immunoprecipitated from cell lysates with anti-Ras-antibodies and after electrophoretic separation in an SDS-polyacrylamide gel and membrane transfer, detected with anti-Ras^{Val12} antibodies. The name ras^{Val12} Std. denotes a standard K-Ras^{Val12} preparation (Oncogene Science).

iv) Inhibition of the IL-3 independent growth of H-ras^{Val12} transfected cells by inhibiting Ras farnesylation

40,000 cells were seeded in 100 µl RPMI-1640 medium without phenol red (BioWhittaker), supplemented with 10% FCS, 2% glutamine, and 50 µM ß-mercaptoethanol, in 96-well plates. Untransfected FDC-P1 cells were cultivated in the same medium with the addition of 0.1% IL-3 containing cell supernatant so that the cells were not forced into apoptosis. A basic solution of the farnesyltransferase-inhibitor L-739.749 (10 mM in DMSO) was serially diluted with PBS with the addition of DMSO and 10 µl of the dilutions were pipetted into the cells and then incubated at 37°C and 5% CO_2. The final DMSO-concentration was 0.5% (v/v) in every case. The number of living cells was indirectly determined by photometric MTS assay (see above). For measuring the DNA synthesis rate, 10 µl of [6-^{3}H]thymidine in PBS (0.1 µCi; 27 Ci/mmol, Amersham) were added to each well 16 h before the measurement. The cells were transferred onto filter plates using a cell harvester (Canberra Packard), washed with water and the quantity of [^{3}H]thymidine incorporated in the DNA was determined in a scintillation counter (TopCount, Canberra Packard). The values shown in Fig. 1 and Fig. 2 correspond to the average from triplicate measurements, based on the untreated cells. (Figs. 1 and 2 show the inhibition of H-ras^{Val12} dependent proliferation (viability, given as relative absorption, in Fig. 1; DNA synthesis in Fig. 2) by farnesyltransferase inhibitor L-739.749 in IL-3-free medium. FDC-P1: IL-3-dependent untransfected starting cells.

FDC-P1/J2: IL-3 and Ras-independent FDC-P1 cell line, transformed with activated v-myc plus v-raf. FHRV12cc21: IL-3 independent FDC-P1 cell line, transformed with human H-ras^{Val12}.)

b) Preparation of KDR test cells

i) Cloning of KDR and preparation of expression plasmids (receptor-DNA)

The cDNA of the human KDR was obtained by screening a cDNA bank and cloned into the expression vectors pRc/CMV (Invitrogen V750-20) or LXSN (Miller, et al., 1989).

ii) Isolation of clones which contain the sequence coding for human KDR

By screening a human endothelial cell cDNA bank in the lambda gt11 vector (Clontech HL 1024b) with PCR-probes obtained by "nested" amplification of two KDR gene fragments (Terman et al., 1992; EMBL Accession Nr. X61656) using the DNA extracted from the same bank as the starting material, 13 clones were isolated and the inserts contained in the plasmid pUC18 were sequenced. The sense primers used for the PCR-amplification corresponded to the amino acid sequences RGQRDLDWLWP, (SEQ ID NO:7) WDSKKGFTIP, (SEQ ID NO:3) GARFRQGKDYVG, (SEQ ID NO:4) and CWHGEPSQRP, positions 52-62, 177-186, 940-951 or 1140-1149. The antisense primers corresponded to the amino acid sequences (SEQ ID NO:5) TLVIQAANVSA, and (SEQ ID NO:6) SGYHSDDTDTT, positions 514-524 or 1305-1315. The full-length cDNA-clone was assembled by ligating three overlapping clones and two pairs of complementary oligonucleotides, coding for the 38 amino acids of the published sequence, which does not go up to the start-ATG, plus four additional amino acids, including the necessary ATG. Nucleotides which promote efficient translation of the protein were inserted upstream of the ATG (GGATCCCTCGACGCGCC, (SEQ ID NO:7) the BamHI site at the beginning of this sequence is used for cloning purposes). It was possible to ligate the synthetic oligonucleotides by creating an EcoRV site at amino acids 37-38 (GAC ATA was mutated into GAT ATC, without changing the sequence for the amino acids DI). The

sequences of the cDNA-clones obtained differ from the published sequence (Terman et al., 1992; EMBL-Datenbank, Accession Nr. X61656) at positions 770 (Ala instead of Thr), 785 (Arg instead of Gly), 846 (Val instead of Glu), and 1345 (Ser instead of Thr). These changes make the sequence obtained at the corresponding positions identical to mouse homologues of KDR, Flk-1 (Matthews et al. 1991; GeneBank Accession No. X59397; Millauer et al., 1993; EMBL Accession No. X70842), except at the last position, which is situated in a poorly conserved region. In addition, two of the amino acids in question, namely 770 and 785, are located in the region which was reported to be the transmembranal domain (amino acids 763 bis 787, Terman et al., 1992). It was therefore necessary to make the edge regions similar to groups 763 to 784, corresponding to Flk-1 (Matthews et al., 1991).

iii) Subcloning KDR-cDNA into the expression vector pRc/CMV

The fully assembled KDR-cDNA was first excised from pUC18-KDR with Acc65 (isoschizomer KpnI) and SalI and subcloned into the Acc65 and SalI site of pAD-CMV2 (EP-A 393 438). Then the resulting plasmid pAD-CMV2-KDR was cut with XhoI, the DNA ends were filled using the Klenow fragment of DNA-polymerase I in the presence of all four dNTPs, extracted with phenol and precipitated with ethanol. The blunt end DNA obtained was digested with XbaI. The expression vector pRc/CMV was cut with HindIII, the DNA ends were made blunt, and the vector was then cut with XbaI. The KDR-sequence having a blunt end and an XbaI end which was excised fom the plasmid pAD-CMV2 was ligated into this vector, and the resulting plasmid named pRc/CMV-KDR.

iv) Cloning the KDR-sequence into the expression vector pLXSN

The expression vector pLXSN was cut with Hpa I. The KDR sequence doubly excised from the plasmid pAD-CMV2-KDR with XhoI and XbaI and then blunted by the addition of all four dNTPs using Klenow enzyme was ligated into this vector. A resultant clone of the correct orientation was designated pLXSN-KDR.

v) Transfection of FDC-P1 cells

1 x 10^7 FDC-P1 cells were centrifuged off at 1200 rpm at ambient temperature, washed once with serum-free RPMI and resuspended in 0.8 ml serum-free RPMI. 20 µg pRc/CMV-KDR, linearised with ScaI, were added and electroporated at 280 V, 980 microfarad, 1 sec. Electroporated cells were resuspended in Iscoves Modified Dulbecco's medium (IMDM) with interleukin-3 (IL-3) and 10% foetal calf serum (FCS) and cultivated. Two days after transfection the selection of transfectants was started by the addition of 400 µg/ml geneticin (G418). Another two days later the G418 concentration was increased to 800 µg/ml and subsequently to 1000 µg/ml. Cells killed off by G418 were separated from resistant cells by centrifugation in a Ficoll gradient. To do this, the mixture of dead and living cells was centrifuged, as described above, resuspended in 1 ml IMDM + IL-3 + 10% FCS + 1 g/l G418 and placed over 1 ml Ficoll (Pharmacia). The gradient was run for 15 min at ambient temperature at a speed of 2500 rpm. The living cells are found in the intermediate phase, whilst dead cells are pelleted. The cells of the intermediate phase are washed twice with 10 ml IMDM + IL-3 + 10% FCS + 1 g/l G418 and further cultivated in the same medium.

In order to obtain G418 resistant cells which grow independently of IL-3, but dependent on VEGF, the IL-3 concentration was reduced to a tenth and at the same time 10 ng/ml of human recombinant VEGF 165 (Fiebich et al., 1993) was added. Dead cells were again separated from the living ones using Ficoll gradients. The IL-3 was reduced to a tenth of the previous IL-3 concentration, until the cells could be kept independently of IL-3.

In order to obtain individual clones, these cells were cloned out in Methocel (Metcalf et al., 1992; Meckling-Gill et al., 1992). The cloning out was carried out in the absence of G418, but in the presence of VEGF.

The cell clones (FDCP-CMV-KDR) were tested for their ability to be stimulated by VEGF. For this, the cells were seeded in 96-well plates (15000 cells/ well) and cultivated with or without VEGF. After 48 hours an MTS assay (Promega Non radioactive cell proliferation assay: Cell Titer 96AQ) was carried out and the cell growth was determined using this colorimetric assay. None of the clones displayed any increased growth in the presence of VEGF. As was demonstrated by RT-PCR (Reverse-transcriptase polymerase chain reaction), FDC-P1 cells and the transfected cells (FDCP-CMV-KDR) themsleves produce VEGF. FDCP-CMV-KDR cells are thus stimulated autocrinally.

vi) Infection of FDC-P1 cells

GP+E-86, a packaging cell line for retroviruses (Markowitz et al., 1988.), was grown in IMDM with 10% FCS in 60 mm culture dishes until 70-80% confluent. These cells were transfected overnight using

lipofectamine reagent (Gibco BRL) according to the manufacturer`s instructions with 5 µg of uncut (supercoiled) DNA (pLXSN-KDR) and 10 µl of lipofectamine. The next day, the cells were detached with trypsin, centrifuged off, taken up in medium, transferred into culture flasks (75 cm^2) and the selection of transfectants was started by the addition of 800 µg G418/ml. After another two days the G418 concentration was increased to 1000 µg/ml. After the selection the GP+E-86-LXSN-KDR cells were kept with 10 ng/ml VEGF. To infect FDC-P1 cells, the resistant GP+E-86-LXSN-KDR cells were pooled and 500,000 cells were seeded in IMDM with 10% FCS without G418 in 60 mm culture dishes. 100,000 - 200,000 FDC-P1 cells were added and co-cultivated for 48 hours in the presence of 1% IL-3. The infected FDC-P1 cells (FDCP-LXSN-KDR) were transferred into culture flasks and kept in the presence of VEGF and 1% IL-3. Two days after co-culture, the selection in G418 was started. The selection, IL-3 reduction and cloning out were carried out as described in v) for the transfection of cells (FDCP-CMV-KDR).

c) Preparation of EGF-R (Epidermal Growth Factor Receptor) test cells

EGF-R-test cells designated F/LHERc cells were prepared from the interleukin-3-dependent haematopoetic mouse cell line $FDC\text{-}P_1$ by infection with a recombinant retrovirus containing the DNA coding for the human EGF-R. The recombinant retrovirus used for the expression of human EGF-R-cDNA (Ullrich et al., 1984) corresponds to the construct described by von Rüden, T. et al., 1988, except that the retroviral vector LXSN (Miller al., 1989) was used for the expression of EGF-R cDNA (as in a) for using

KDR) and the packaging cell used was the line GP+E86 (Markowitz al. 1988). Selection and cloning of the cells were carried out essentially as described in a). In order to select virus-infected FDC-P1 cells the cultures were selected out in 1000 µg/ml G418 in the presence of 1% IL-3 (1% conditioned medium of the IL-3-secreting cell line X63-0 mIL-3; Karasuyama and Melchers, 1988), in order to ensure maximum IL-3 stimulation, as the virus construct also carries a neomycin resistance gene next to the human EGF-R cDNA. Then 20ng/ml human recombinant EGF (Promega) was added to the cultures of G418-resistant cells and the IL-3 concentration was successively brought back from 1% to zero. Cells which grew EGF-dependently in the absence of IL-3 were finally cloned in methylcellulose in the presence of TGF-α, but not IL-3 (like EGF, TGF-α also stimulates the EGF receptor, but is more stable). Finally it was confirmed, with each isolated clone, that the cells die in the absence of EGF and IL-3, that they proliferate in the presence of EGF or IL-3 as the sole growth factor, and that the EGF stimulation is cancelled out by known EGF receptor inhibitors.

d) Preparation of HER2 test cells

i) Subcloning of the mutant HER2 sequence into the expression vector pAHygCMV1

From plasmid pHer2-CVN, which contains the complete 4.5 kb coding sequence of the human receptor HER2 (GeneBank AC-No. M11730; Coussens et al., 1985), the HER2-cDNA was excised in 2 parts and subcloned: First the 3.2 kb SalI-KpnI fragment which contains the 5´-part of the HER2-cDNA was excised and cloned in a directed manner into the expression vector pAHygCMV1 doubly cut with SalI and KpnI, so that the transcription of HER2 is under the control of the cytomegalovirus (CMV) promoter/enhancer

element. The resulting intermediate product was cut first with NotI, the ends were made blunt ("blunt ended") by the addition of dNTPs and the Klenow fragment of DNA-polymerase, and then cut with KpnI. Then the 0.8 kb KpnI-StuI fragment which contains the 3´-part of the HER2-cDNA was excised from pHer2-CVN and directedly cloned into the intermediate construct. The expression vector pAHygCMVHer2 formed contains the complete coding region of HER2 under the control of the CMV promoter.

In order to introduce the point mutation Val659->Glu659 (GTT->GAA) (Suda et al., 1990) the 3.2 kb SalI-KpnI HER2-fragment was first cloned into the plasmid pBluescript KS (Stratagene), which had previously been opened with SalI and KpnI. For the mutagenesis, an oligonucleotide which contains the AatII cutting site and, 16 bp downstream, the mutated sequence GAA, and an antisense oligonucleotide which contains the sequences around the NdeI cutting site, were synthesised. Using these two oligonucleotides as primers the 400 bp AatII-NdeI fragment was amplified by PCR (polymerase chain reaction) from the SalI-KpnI HER2 fragment which now contains the mutation GTT->GAA. The amplified fragment was recut with AatII and NdeI and cloned into the plasmid pBluescript/Her2(SalI-KpnI) which had also been cut with AatII and NdeI. The region from AatII to NdeI was sequenced to ensure that no mutations other than those desired were obtained as a result of the PCR amplification. Plasmid pBluescript/Her2(V659E) now contains the mutated form of the 3.2 kb SalI-KpnI HER2-fragment. This mutated fragment was re-excised from the plasmid with SalI and KpnI, the non-mutated fragment was also excised from the expression vector pAHygCMVHer2 with SalI and KpnI, and replaced by the mutated fragment. The resulting expression vector, known as pAHygCMVHer2(V659E), now contains the complete coding

region of the mutated, constitutively active form HER2(V659E).

ii) Transfection of the FDCP cells

FDC-P1 cells (Dexter et al., 1980), which grow IL-3-dependently, were transfected with plasmid pAHygCMVHer2(V659E) as follows: About $1x10^7$ cells were mixed with 10 μg plasmid DNA in IMDM-medium (Bio-Whittaker) and electroporated with an Electroporator (Hoefer, Progenetor II) at 980 μF, 280 V for 1000 ms. The cells were kept in IMDM-medium in the presence of 10% heat-inactivated foetal calf serum, 50 μM ß-mercaptoethanol, standard antibiotics, and 1% IL-3 at 37°C under 5% CO_2 and selected by the addition of 300 mg/l hygromycin B (Boehringer Mannheim). After successful selection the cells were slowly weaned off IL-3, then the IL-3-independently growing cell pool was subcloned in Methocel. Individual clones were picked and cultivated in medium without IL-3.

iii) Testing the usefulness of the cells

Individual clones were tested in a fluorescence-activated cell sorter (Becton-Dickinson, FACSort) for surface expression of HER2, using as the primary antibody a mouse antibody directed against the extracellular domain of HER2 (Oncogene Science, c-neu (Ab-2)), and as the secondary antibody a fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Dianova, Hamburg). The subclone with the best surface expression of HER2(V659E), known as FDCP/Her2-C42#16, was used for the screening.

e) Preparation of HGF (Hepatocyte Growth Factor) receptor test cells

i) Subcloning of the c-met sequence into the expression vector pLXSN

The complete 4.6 kb coding sequence of the human HGF receptor c-met (GeneBank AC-No. J02958; Park M., et al., 1987), was directedly cloned into the expression vector pLXSN doubly cut with EcoRI and XhoI. Plasmid pLXSN is a retroviral vector which is used to produce recombinant retroviruses (Miller, et al. 1989). The expression vector pLXc-met formed contains the complete coding region of c-met.

ii) Transfection of the packaging cell line GP+E86

The packaging cell line GP+E86, a mouse fibroblast cell line (Markowitz et al., 1988), was transfected with plasmid pLXc-met as follows: About $1x10^6$ cells per 10 cm plate were seeded in DMEM-medium (Bio-Whittaker) in the presence of 10% heat-inactivated foetal calf serum (FCS) in a number of plates. The next day 20 μg of plasmid-DNA was precipitated with calcium phosphate and the precipitate was slowly added dropwise to the cells. After 4 hours the cells were incubated for 2 min in 15% glycerol, washed and then kept in DMEM-medium in the presence of 10% heat-inactivated foetal calf serum and standard antibiotics at 37°C under 5% CO_2. The cells were selected by the addition of 1 g/l G418 (GIBCO BRL). Cell clones were pooled and the cell pool was cultivated.

The cell supernatant should contain replication-defective recombinant virus particles which infect cells of murine origin. In order to determine the virus titre, $5x10^4$ NIH-3T3(tk^-) cells were seeded per 6 cm dish and incubated

with various concentrations of the cell supernatants (1 ml supernatant + 8 μg/ml Polybren). Cells were selected in G418 and the virus titre was determined from the number of clones.

iii) Infection of the FDC-P1 cells

FDC-P1 cells (Dexter et al., 1980) which grow IL-3-dependently were infected with recombinant virus particles as follows: About $2x10^6$ cells were incubated in 2.5 ml virus supernatant of the GP+E86/LXc-met cells and 8 μg/ml of Polybren and 1% IL-3 for 4 hours at 37°C. The cells were diluted in IMDM-medium in the presence of 10% heat-inactivated foetal calf serum, 50 μM ß-mercaptoethanol, standard antibiotics and 1% IL-3 and kept at 37°C under 5% CO_2. The selection was carried out by the addition of 1 g/l G418 (GIBCO BRL). Antibiotic-resistant stable cell lines were then selected for HGF-dependent and IL-3-independent growth by adding HGF and removing IL-3. To do this, the cell pool still growing dependent on IL-3 was first subcloned in Methocel. Clones which were still IL-3-dependent clones were picked out and individually slowly weaned off IL-3 by keeping the clones in 20 ng/ml recombinant HGF (Sigma) as growth factor while the IL-3 concentration was reduced more and more. Individual HGF-dependent but IL-3-independent clones were cultivated.

iv) Testing the usefulness of the cells

In order to establish whether the clones grow HGF-dependently, the cells were seeded in various concentrations of HGF and the growth of the cells was determined by the incorporation of ^{3}H-thymidine: For this, cells were seeded in microtitre plates (at a concentration of 20000 cells per well) and incubated with various concentrations of recombinant HGF (Research &

Development). After 2 days ^{3}H-thymidine was added overnight and the incorporation of thymidine was determined; in addition, as stated in Example 1a), the number of living cells was determined indirectly by a photometric MTS assay using absorption (Fig. 4) measured the next day (Packard, TopCount). Positive clones were additionally tested in a fluorescence-activated cell-sorter (Becton-Dickinson, FACSort) for surface expression of c-met, using as primary antibody a murine antibody directed against the extracellular domain of c-met (Upstate Biotechnology, Inc., Cat.# 05-237), and as secondary antibody a fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Dianova, Hamburg). The subclone with the best surface expression of c-met, known as FDCP/LXc-metH6, was used for the screening.

f) High Throughput Screen

The test cells obtained according to a) to e) were cultivated in RPMI/1640 medium (BioWhittaker), supplemented with 10% foetal calf serum (FCS; Boehringer Mannheim), 2 mM glutamine (BioWhittaker), at 37°C and 5% CO_2, in the presence of the corresponding growth factors. In order to investigate the inhibitory activity of the compounds being studied (test substances) 1.5 x 10^4 cells were cultivated in 100 µl medium per well in 96-well plates in the abovementioned medium with the addition of the corresponding growth factors. The chosen test substances were present in a concentration of 5 mg per ml dissolved in 100% dimethylsulphoxide (DMSO) and were added to the cultures in a dilution of 1:1000, whilst the DMSO concentration did not exceed 0.1%. The final concentration of the test substances was 5 µg/ml in the test system, each substance was tested in 3 microtitre plate wells (triplicate measurement). On each plate three positions were treated only with DMSO in the corresponding dilution

(1:1000); these positions were used as a negative control. The cultures were incubated for 21 h at 37°C under cell culture conditions. After the end of the incubation period 20 µl of a mixture of MTS (0.88 mg/ml; Promega G511A) and PMS (0.11 mg/ml; Sigma P-9625) were added and incubated for a further 3 hours at 37°C. Once the incubation period had ended, the test plates were measured in a microtitre plate photometer and the data were evaluated in a data bank. The relative cell number which correlates with the extinction at 492 nm was given as a percentage of the negative control of the test plate.

g) Comparative screening and testing for specificity

The High Throughput Screen described in f) was carried out parallel to the test cell lines described in a) to e). The same dilutions of the test substance were simultaneously added to the cells placed in various microtitre plates, which were subsequently kept under exactly the same conditions and evaluated. Substances which, in a test system, had a value of less than 50% of the negative control value on the same plate, were regarded as potential hits and investigated more closely for specificity. A substance was regarded as specific if, in the assay in question, it had a value of less than 50% of the negative control and in the parallel assays achieved a value of more than 75% of the corresponding negative control. These substances were selected and examined in a detailed dosage-activity curve for their IC50-value. The cells were placed in a microtitre plate under the same conditions as described in f) and investigated with a serial dilution of the corresponding test substance in concentrations of between 400 µM and 10 nM. The IC50 value is the concentration of test substance in which the test value reaches 50% of the negative control. Table 1 shows a

summary of the results obtained from tests with a larger quantity of test substances.
A total of 231360 tests yielded 2834 "hits", i.e. substances which satisfy the criterion of "inhibition" to less than 50% of the control, corresponding to a percentage of 1.22% of the substances tested. As a rule, in screening to find active substances, the concentrations of the test substances are adjusted so that about 1-2% of the test substances meet the criteria specified. These substances then have to be investigated manually and with considerable effort for their specificity and mechanism of activity. This is generally done by detailed tests with various cellular and enzymatic assays. In high throughput screening this would represent substantial labour and other costs. The parameters set by the comparative screening according to the present invention narrow down the range of interesting substances needing investigation in detailed secondary assays to about 10% of the original hits. After detailed investigations 13 substances which selectively block the target molecule in question still remained (4.3% of the hits regarded as specific), constituting a guiding structure for the development of active substances.

The specificity of an inhibitor for the EGF-R is illustrated by the example of 4-[(3-chlorophenyl)-amino]-6-(cyclopropylamino)-pyrimido-[5,4-d]pyrimidine in Fig. 7. Whereas the IC50 value for the EGF-dependent cell line (in Fig.7: "EGF auto5") is about 500 nM, on the other test cell lines containing Ras (in Fig.7: "FHR V12"), HER-2 or HGF-R (in Fig.7: "TPRmet") as target molecules, effects on the proliferation of the cells can only be measured at a concentration 100 times greater. The failure rate of 95% after detailed tests can be explained by the fact that the test cells do indeed have an identical genetic background, but are clonal in origin, on the one hand, and ectopically

express different target molecules, on the other hand. As a result there are slight differences in sensitivity compared with nonspecifically active organic substances.

Example 2

Luciferase assay in the High Throughput Format for screening substances with a specific effect on receptor-tyrosinekinase-dependent signal transmission pathways

a) Preparation of recombinant KB cells, which express luciferase as a function of HGF or EGF.

i) Subcloning of the c-fos promoter region into the expression vector pBHlucOL

The promoter region of the human c-fos gene was excised from plasmid pfosCAT (Schönthal, A. et al., 1988) with XbaI and HindIII and subcloned first into plasmid pBluescript SK (Stratagene). The resulting intermediate product was first cut with XbaI, the ends were made blunt ("blunt ending") by the addition of dNTPs and the Klenow fragment of DNA-polymerase and then cut with SalI. Plasmid pBHlucOL, which contains the luciferase gene (Voraberger, G. et al., 1991), was cut first with HindIII, the ends were made blunt by the addition of dNTPs and the Klenow fragment of DNA-polymerase, then cut with SalI, and the fos-promoter fragment was cloned into this, resulting in plasmid pBHfosluci.

ii) Transfection of the KB cells

The human epidermoid carcinoma cell line KB (ATCC CCL 17) was transfected with plasmid pBHfosluci as follows: about $1x10^6$ cells per 10 cm plate were seeded in DMEM-medium

(Bio-Whittaker) in the presence of 10% heat-inactivated foetal calf serum in several plates. The next day 20 µg of plasmid-DNA were precipitated with calcium phosphate and the precipitate was slowly added dropwise to the cells. After 4 hours the cells were incubated for 2 min in 15% glycerol, washed and then kept in DMEM medium in the presence of 10% heat-inactivated foetal calf serum and standard antibiotics at 37°C under 5% CO_2. The cells were selected by the addition of 0.5 g/l G418 (GIBCO BRL). Individual G418-resistant cell clones were cultivated and tested for the inducibility of luciferase expression by the addition of HGF (Sigma). Approximately 10000 cells of each clone were seeded 6-fold in 200 µl per well in a light-impermeable 96-well microtitre plate covered with tissue culture (Microlite™, Dynatech Laboratories) and incubated overnight at 37°C. Three batches were treated with 20 ng/ml HGF and incubated for a further 6 h. Then the medium was removed and the cells were washed twice with PBS. The cells were taken up in 150 µl of lysing buffer (25 mM Tricin, 0.5 mM EDTA, 0.54 mM sodium tripolyphosphate, 6.5 mM DTT, 16.3 mM $MgSO_4.7H_2O$, 0.1% Triton X-100, 1.2 mM ATP, 0.05 mM luciferin; pH7.8) per batch and the luciferase activity was measured in a 96-well luminometer (ML-1000, Dynatech). Cell clone KB-B4 was chosen for further experiments, as it had a measurable base level of luciferase activity as well as a 10-15-fold inducibility by HGF.

iii) Testing the usefulness of the cell line KB-B4

In order to determine whether the luciferase gene in clone KB-B4 can be stimulated by HGF or EGF the cells were seeded in various concentrations of HGF or EGF and the luciferase activity was measured: For this the cells were seeded in microtitre plates in a concentration of 10000 cells per well and incubated with various

concentrations of HGF or EGF. After 6 hours the cells were lysed as above and the luciferase activity was measured. A dosage-dependent induction was apparent, with a maximum induction of 10- or 20-fold. An Example is shown in Fig.5.

Since various inhibitors of tyrosinekinase activity of the EGF receptors are known, KB-B4 cells were incubated for 6 hours with various concentrations of different inhibitors with the addition of 1 ng/ml of EGF and then the luciferase activity was measured. All the inhibitors tested produced a dosage-dependent inhibition of luciferase induction by EGF. An example of the inhibitory activity of an inhibitor is shown in Fig.6, taking the example of the test substance 6,7-dimethoxy-4-(3-methylphenylamino)quinazoline.

b) High Throughput Screen

The KB4 cells described in a) for testing for EGF and HGF were propagated in DMEM (Bio Whittaker), supplemented with 10% FCS and 500 mg/ml G418. In order to investigate the test substances, 1 x 10^4 cells were seeded in 100 μl phenol-red-free DMEM/1% FCS per well in 96 well microtitre plates. Chosen test substances were present in a concentration of 5 mg per ml dissolved in 100% dimethyl sulphoxide (DMSO) and were added to the cultures in a dilution of 1:600, the DMSO concentration not exceeding 0.25%. The final concentration of the test substances was about 8.3 μg/ml in the test system, each substance was tested in three microtitre plate wells (triplicate measurement). On each plate three positions were treated only with DMSO in the corresponding dilution (1:600); these positions were used as a negative control. After the addition of the test substances the test cells were induced with EGF (20 ng/ml) or HGF (20 ng/ml) in order to

induce the fos promoter. After an incubation period of 5 h at 37°C and 5% CO_2, 150 μl of lysing buffer (25 mM Tricin, 0.5 mM EDTA, 0.54 sodium tripolyphosphate, 16.3 mM $MgSO_4$, 1.2 mM ATP, 0.05 mM luciferin, 56.8 mM ß-mercaptoethanol, 0.1% Triton X-100, pH 7.8) were added and the luminescence was measured in a Wallac Microbeta and the data were evaluated in a data bank. The relative promoter activity, which correlates with the luminescence measured, was given as a percentage of the negative control of the test plate.

c) Comparative screening and testing for specificity

The High Throughput Screen described in b) was carried out parallel to the two cell lines mentioned above. The same dilutions of the test substance were added simultaneously to the cells placed in various microtitre plates, which were subsequently kept under exactly the same conditions and evaluated. Substances whose results were less than 50% of the negative control in one test system and more than 70% of the negative control in the parallel assay were regarded as specific inhibitors of the test systems in question. These substances were tested a second time in a corresponding High Throughput Screen in order to check the reproducibility. Substances whose results were repeatable were tested for their IC-50 value in a detailed dosage-activity curve. The cells were placed in a microtitre plate under the same conditions as described in b) and tested with a serial dilution of the corresponding test substance in concentrations between 400 uM and 10 nM. The IC50 value is the concentration of the test substance at which the test value reaches 50% of the negative control. Tab. 1 shows a summary of the results obtained from tests with 2676 substances.

Table 1

Assay	Hits	Hits %	Specific hits	specific hits in %
EGF	28	1.04	7	0.26
HGF	27	1.00	6	0.22

Example 3

Luciferase assay in the High Throughput Format for screening substances with a specific effect on signal transmission pathways which are dependent on G protein-coupled receptors

a) Preparation of three different recombinant A549 test cell lines which express luciferase as a function of the activation of the human neurokinin-1(NK1) receptor, the neurokinin-2(NK2) receptor or the serotonin receptor $5HT_2$

The preparation and cultivation of the NK2 cell line (A20/NK2-122) and the $5HT_2$ cell line were carried out as described in Example 6 of WO 93/11257. In order to prepare the NK1 cell line (A20/NK1-12A) the cDNA of the human NK1 receptor (Gerard et al., 1991) was cloned into the expression vector pAHygCMV1 analogously to NK2 and in this way the pretest cell line was transfected. The rest of the procedure and the cultivation were carried out in the same way as for the NK2 test cells.

b) High Throughput Screen

The cell lines prepared in a) for testing for NK1 and NK2 are propagated in RPMI/1640 medium (BioWhittaker), supplemented with 10% dialysed FCS, 0.8 g/l G418 and 0.15 g/l hygromycin. To investigate the test substances $1x10^4$ cells are seeded in 100 µl of UltraCulture medium (BioWhittaker) per well in 96 well microtitre plates. Chosen test substances were present in a concentration of 5mg/ml dissolved in 100% dimethylsulphoxide (DMSO). They were added to the cultures in two concentrations of 1:300 and 1:3000, with the DMSO concentration not exceeding 0.5%. The final concentration of the test substances was about 16.6 and 1.66 µg/ml in the test system, with each substance being tested in four microtitre plate wells per dilution. On each plate four positions per dilution were treated only with DMSO in the corresponding dilution (1:300, 1:3000). These positions were used as a negative control. After the addition of the test substances, the NK1 cells were mixed with substance P (1 µM) to induce the TRE-sensor-DNA, the NK2 cells were mixed with neurokinin A (10 µM) and the $5HT_2$ cells with serotonin (100 µM). After an incubation period of 7 h at 37°C and 5% CO_2 the microtitre plates were washed 4 times with 150 µl of washing buffer per well (25 mM Tricin, 16.3 mM $MgSO_4$). Then 100 µl lysing buffer (25 mM Tricin, 0.5 mM EDTA, 0.54 mM sodium tripolyphosphate, 16.3 mM $MgSO_4$, 1.2 mM ATP, 0.05 mM luciferin, 56.8 mM ß-mercaptoethanol, 0.1% Triton X-100, pH 7.8) were added and the luminescence was measured in a Dynatech Luminometer and the data were evaluated in a data bank. The relative promoter activity which correlates with the luminescence measured was given as a percentage of the negative control of the test plate.

c) Comparative screening and testing for specificity

The High Throughput Screen described in b) was carried out parallel to the three cell lines mentioned above. The same dilutions of the test substances were simultaneously added to the cells present in various microtitre plates, which were subsequently kept under exactly the same conditions and evaluated. Substances whose results were less than 40% of the negative control in one test system and were more than 65% of the negative control in the other parallel assays were regarded as specific inhibitors of the test systems in question. Such substances were tested a second time in a corresponding High Throughput Screen to check their reproducibility. Substances whose results were repeatable were selected and subjected to additional tests. Some substances also inhibit two of the three test systems, some nonspecifically inhibit all three assays carried out in parallel. Table 2 shows a summary of the activity of 1458 substances on the tests described.

Table 2

Assay	Number of hits	in %
only NK1, not NK2, 5HT2	5	0.34
only NK2, not NK1, 5HT2	18	1.23
NK1 and NK2, not 5HT2	6	0.41
only 5HT2, not NK1, NK2	74	5.07
NK1, NK2 and 5HT2	61	4.18

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